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Broadleaf trees mediate chemically the growth of Chinese fir through root exudates

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Highlights

• Root exudates chemically mediate Chinese fir growth in species-specific fashion.

• Chinese fir changes root placement pattern in response to heterospecific neighbors.

• Root exudates can influence the composition of main microbial groups.

• Mixing with certain broadleaf species can enhance the performance of Chinese fir.

Abstract Tree performance in mixed-species forest plantations is ultimately the net result of positive and negative interactions among species. Despite increasing knowledge of interspecific interactions, relatively little is known about the chemical mechanisms mediating such interactions. We constructed **mixed planting systems with two species** including the conifer Chinese fir **(*Cunninghamia lanceolata*（Lamb.）Hook)** and broadleaf species ***Cinnamomum camphora* L. Presl, *Elaeocarpus decipiens* Hemsl, *Liquidambar formosana* Hance or *Michelia macclurei* Dandy**. Based on a series of manipulative experiments, we investigated the performance of Chinese fir and analyzed root placement patterns and **the composition of main soil microbial groups**. The broadleaf trees influenced the growth of Chinese fir roots more than the growth of shoots. Furthermore, *C. camphora* roots released allelochemicals into the soil environment, resulting in growth inhibition of Chinese fir and **changes in main soil microbial groups**. However, when grown with *E. decipiens* and *M. macclurei*, the growth of Chinese fir was consistently **promoted**. It **responded by enhancing its root growth and altering root behaviour**, resulting in a shift from growth inhibition to chemical facilitation. **These positive inter-specific interactions** also **stimulated changes in the composition of soil microbes**. **Complementation experiments indicated that non-toxic signaling molecules** in the root exudates of *E. decipiens* and *M. macclurei* may be responsible for mediating positive root-root interactions and **regulating the composition of main soil microbial groups**. Thus, our study demonstrated that broadleaf species chemically mediate the growth of Chinese fir through root exudates. Such a **novel** mechanism

offers many implications and applications for **reforestation programs undertaken to rehabilitate forest plantations that suffer from problems related to the selection of trees**.

Keywords Root exudates; Allelochemicals; **Non-toxic signaling molecules**; Root traits; Root placement pattern; Soil microbial community

Introduction

Managed tree plantations are usually characterized by densely planted monocultures that cause problems, including declined productivity, and reduced biodiversity and ecological services (Kong et al. 2008; Felton et al. 2010; Braun et al. 2017). To mitigate these problems, replacing monocultures with mixed-species plantation forests has become a successful afforestation strategy (Forrester et al. 2006; Griess and Knoke 2011; Liu et al. 2018). Mixed-species plantation forests that are based on a cautious selection of species show clear potential for sustainable and productive forestry. There is a growing interest to reveal the underlying mechanisms, one of the explanations being that a higher diversity of tree species increases the number of ecological niches from the point of view of resource utilization (Forrester et al. 2006; Richards et al. 2010). However, the related chemical mechanisms and optimal combinations of species with particular biochemical traits are largely unknown. **Recent research on belowground ecology has attempted to reveal fascinating and complex interactions, in particular in the rhizosphere. Root exudates serve numerous functions to control biotic and abiotic process** (Chen et al. 2012; Pierik et al. 2013). **These** bioactive metabolites vary substantially among plant species and largely influence intra- and inter-specific plant-plant interactions (Mommer et al. 2016; Tsunoda et al. 2017). Plant-plant interactions mediated by allelochemicals are presumed to represent allelopathy, i.e. negative effects of one plant on another through allelochemical production and release (Inderjit et al. 2011). However, specific root

exudates comprise not only allelochemicals but also a diverse set of secondary metabolites, some of which have been explicitly shown to mediate root-root recognition and trigger changes in root behaviour, possibly through **non-toxic signaling molecules** (Bais et al. 2006; Caffaro et al. 2011; Semchenko et al. 2014; Rasmann and Turlings 2016). Recent studies have investigated species-specific effects of root-root interactions and root placement patterns in mixed-species systems (Belter and Cahill 2015; Kong et al. 2018). Root placement patterns of target plants are highly dependent on the species identity of neighboring roots (Weidlich et al. 2018). Furthermore, plants are able to distinguish between the roots of their own and different species. The generally accepted view is that root exudates play a dominant role in mediating root-root interactions (Chen et al. 2012; Pierik et al. 2013). In a relatively early report, the roots of the desert shrub *Larrea tridentata* were found to inhibit the roots of *Ambrosia dumosa* in their vicinity through releasing allelochemicals (Mahall and Callaway 1991). Root-placement patterns and root-root recognition could also be mediated through root-secreted **non-toxic signaling molecules** (Chen et al. 2012; Semchenko et al. 2014). Our previous studies have found that Chinese fir **(*Cunninghamia lanceolata* (Lamb.) Hook)** proliferates dense and abundant roots towards the roots of ***Michelia macclurei* Dandy**. Interestingly, when applying activated carbon into the soil, this phenomenon disappears due to the tremendous capacity of activated carbon to absorb **non-toxic signaling molecules** (Xia et al. 2016).

111 Once a plant releases bioactive molecules into the soil, a series of abiotic and biotic
112 actions take place. Allelochemicals and **non-toxic signaling molecules** may shape
113 microbial communities and **regulate the growth of associated** beneficial mycorrhizal
114 species (Cantor et al. 2011; Xia et al. 2015, 2016; Rasmann and Turlings 2016;
115 **Majewska et al. 2018**; Zhou et al. 2018). Such specific alterations may result in a
116 positive or negative feedback effect on plant performance (Chaparro et al. 2012; Xia
117 et al. 2016; Guo et al. 2019). However, relatively little is known about plants'
118 interactions with tree-derived bioactive molecules and soil microbial communities.
119
120 The conifer Chinese fir is a **native** and fast-growing tree that accounts for 20-30% of
121 the total commercial timber production in China. **Chinese fir plantations usually**
122 **involve monocultures that cause problems due to replant disease** (Kong et al.
123 2008; Liu et al. 2010; Chen et al. 2014). It has been suggested that establishing mixed
124 broadleaf and conifer plantation forests may be helpful for the maintenance and
125 improvement of Chinese fir productivity (Wang et al. 2008; Xia et al. 2016). However,
126 how to choose suitable broadleaf tree species for **restoring** the Chinese fir plantation
127 forests is still **unclear**. We anticipated that the performance of Chinese fir would be
128 influenced by chemical effects from specific broadleaf tree species, primarily through
129 ecological belowground interactions mediated by root exudates. In this study, we set
130 up a series of manipulative experiments to evaluate the performance of Chinese fir
131 seedlings along with analyses **on** the root distribution and **the composition of main**
132 **soil microbial groups**. We tested the hypotheses that (i) broadleaf trees influence the

133 root growth and behavior of the conifer Chinese fir in a species-specific way, and (ii)
134 the performance of Chinese fir in particular species combinations is influenced by
135 specific root exudates produced by broadleaf trees and by the relationship with soil
136 microbiota.
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Materials and methods

Plant materials and soils

Chinese fir and four broadleaf species, ***Cinnamomum camphora*** **L. Presl,** ***Elaeocarpus decipiens*** **Hemsl,** ***Liquidambar formosana*** **Hance and** ***M. macclurei*** **Dandy**, were selected to be investigated **in** this study. The four broadleaf trees are native of southern China and commonly used in the **establishment** of mixed-species plantation forests with Chinese fir. Their seeds and seedlings were obtained from the Huitong Experimental Station of Forest Ecology, Chinese Academy of Sciences (26°40′ - 27°09′ N, 109°26′ - 110°08′ E; elevation 300 - 1000 m), which is located in the transition zone from the Yunnan-Guizhou plateau to the low mountains and hills on the southern side of the Yangtze River. Soil was collected randomly **from a Chinese fir plantation** at the Huitong Experimental Station. The experimental **soil is Typic Dystrudept** with pH 4.73, soil organic matter content of 26.61 g kg^{-1}, total N of 1.42 g kg^{-1}, available P of 1.68 mg kg^{-1} and available K of 62.82 mg kg^{-1}. Soil samples were air-dried and passed through a 2-mm sieve to remove plant tissues and then used for the series of experiments as described below.

Greenhouse experiments

The first experiment was designed to evaluate root production and root placement

patterns of Chinese fir in the presence of each broadleaf species. The seeds of Chinese fir and broadleaf trees were sterilized with 0.5% $KMnO_4$, then placed into Petri dishes (9-cm diameter) with moistened filter paper for vernalisation in a 4 °C refrigerator for 24 h. All seeds were pre-germinated in a dark chamber at a temperature of 28 °C. **To observe root systems, we used a visual window rhizobox made of two 200 by 400 mm Plexiglas sheets (one black, one clear) and side spacers (40 mm), which separated the two Plexiglas sheets creating the soil space. This configuration was held together with binder clips along the sides. A row of 3 mm holes was prepared at the bottom of each window box to allow for drainage. The system provided soil space for plant growth.** Chinese fir trees were grown in a monoculture or mixed with each broadleaf species in the window rhizoboxes containing 1500 g soil. Each treatment consisted of five replicates. A total of 25 window rhizoboxes were used in this experiment. A single Chinese fir seed with a neighbor was sown into given positions, 1/4 of the space away from the edge. Window rhizoboxes were placed in racks at an angle of 40° with the transparent plexiglass covered with aluminum foil facing down and away from the light source. The angled position could promote more root-plexiglass contact to aid visual observations. All window rhizoboxes were watered every 2 d until the final harvest.

After seven months (from March to September 2016), the window rhizoboxes were opened. Chinese fir seedlings in each treatment were harvested for the above- and belowground biomass measurements. We constructed six metrics for the Chinese fir

199 roots to show their responses to the presence of broadleaf trees or another Chinese fir.
200 The root systems were scanned to yield a gray-scale TIFF image. The image was
201 analyzed with WINRHIZO (Regent Instruments, Quebec, Canada), after which the
202 roots were oven-dried for biomass measurements. From each analysis, six root
203 parameters were used, including a size-related metric (total root length), three
204 measures of habitat occupancy (total root **occupation** area, maximum root **amplitude**
205 and maximum root depth) and two architecture measures (horizontal asymmetry in
206 root length or root biomass). **Details of each measure are supplied in Table S1.**
207
208 The purpose of the second experiment was to evaluate the impact of belowground
209 segregation on mixed-species patterns of Chinese fir with broadleaf trees. A series of
210 18 (diameter) × 16 cm (height) plastic pots with 5 kg soils were used in this
211 experiment. **Two seedlings were planted into each pot, including Chinese fir**
212 **mixed with each broadleaf species or another Chinese fir. Then, the pots were**
213 **divided into three groups.** The first group was not exposed to any treatment, while
214 the other two groups were segregated with 30-μm nylon mesh or plastic film in the
215 middle of the pot, resulting in two sets of root-root interactions. The no-treatment
216 plants had root contact or soil exchange between Chinese fir and broadleaf trees. The
217 30-μm nylon mesh prevented the penetration of root systems but allowed chemical
218 and microbial interactions in the pots. The plastic film completely blocked root and
219 soil interactions between Chinese fir and broadleaf trees. We then exposed
220 one-year-old seedlings of Chinese fir and each broadleaf species into 15 treatments

(Chinese fir monocultures serving as controls). One seedling of each species was planted 5 cm apart in each pot. Each treatment consisted of four replicates. All plots were watered every other day and randomized once a week. The experiment began in March and ended in September 2017. In the 30-μm nylon mesh segregation treatment, the soil adhering to roots was defined as rhizosphere soil. The soils were freeze-dried and used for the determination of **main soil microbial groups** utilizing the phospholipid fatty acid (PLFA) method, as described in Xia et al. (2015). Then, the roots were oven-dried for biomass measurements.

The third experiment was conducted to examine species-specific effects of root exudates on Chinese fir root growth. Root exudates from each broadleaf species and Chinese fir were collected with a specially made continuous root exudate trapping system (CRETS). The CRETS system is a hydroponic device with a steel structure. In the greenhouse, one hundred seedlings of each tree species were transplanted into the container. Chemical trapping was started after the column (5 × 25 cm) was packed with 250 g Amberlite XAD-4 resin (Aldrich Co., USA). After 30 days, the column was removed. The resin column was continuously washed with deionized water for 24 h to clear inorganic ions and carbohydrates. Then, the resin was eluted with methanol and the root exudates were obtained after removing methanol. **After that, the collective root exudates were divided into two parts defined by the ratio of 4:1. Each part was dissolved in 300 ml water and stored at -20 °C.**

We transplanted one-year-old Chinese fir seedlings into pots (15 × 20 cm) with one seedling per pot. We filled 44 pots with 5 kg of soil and placed them in a greenhouse in a completely randomized design with four replicates for each treatment. A week after transplanting, **we separately took 10 ml from each original solution of each species at two doses and diluted it into 2 L water (four replicates, each pot treated with 500 ml) to treat Chinese fir seedlings every 7 days. The same volume of deionized water was applied as a control. Thus, there were three doses of root exudates from each species at 100%, 25% and 0% (control) dilutions, which were used for treatments 20 times during the growing season.** All pots were irrigated with tap water every other day and randomized once a week. Chinese fir seedlings were used for biomass measurements after seven months (from March to September 2018). All experiments were conducted in a glasshouse at the Hangzhou Normal University in Zhejiang. The temperature in the glasshouse was maintained at 21-25 °C during the day and 15-18 °C at night, with 12-14 h daytime throughout the growth period.

Environmental chamber experiments

The fourth experiment was performed to evaluate the impact of root exudates on **main soil microbial groups**. It was conducted in controlled environmental chambers (1 m^3), each in a completely randomized block design with three replicates. In all, 36 vials (150 ml) with 100 g soil were pre-cultured in the dark at 28 °C for 10 days. After that,

10 ml of root exudates with deionized water at 1/1 from each species (four broadleaf species and Chinese fir) were applied to 30 vials. Other 6 vials received only deionized water as control pots. The vials were airtight, placed in a chamber at 28 °C, and aerated once a day for 1 h. Vials were taken from the chamber randomly after 3 and 9 days, and the soils were used for the PLFA analysis of **the main soil microbial groups**.

Soil microbiological analysis

A total of 22 PLFAs were identified in the soil samples. Among them, the fatty acids present in proportions >0.5% were included in the analysis. The following biomarkers were used: saturated fatty acids (i14:0, a14:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0, cy17:0 ω7c, cy19:0 ω7c, 16:0 10-methyl, 17:0 10-methyl, 18:0 10-methyl, 14:0, 15:0, 15:0 DMA, 16:0, 17:0, 18:0 and 20:0), monounsaturated fatty acids (16:1 ω9c, 16:1 ω7c, i17:1 ω9c, 17:1 ω8c, 18:1 ω7c, 18:1 ω5c, 18:1 ω9c and 16:1 ω5c), polyunsaturated fatty acids (18:2 ω6c); Gram-positive bacteria (i14:0, a14:0, i15:0, a15:0, i16:0, a16:0, i17:0 and a17:0), Gram-negative bacteria (16:1 ω9c, 16:1 ω7c, i17:1 ω9c, 17:1 ω8c, 18:1 ω7c, 18:1 ω5c, cy17:0 ω7c and cy19:0 ω7c), saprophytic fungi (18:1 ω9c and 18:2 ω6c), arbuscular mycorrhizal fungi (AMF) (16:1 ω5c) and actinomycetes (16:0 10-methyl, 17:0 10-methyl and 18:0 10-methyl). The sum of the Gram-positive bacteria (Gram +), Gram-negative bacteria (Gram -) and non-specific bacteria (14:0, 15:0, 15:0 DMA, 16:0, 17:0, 18:0 and 20:0) was used as total bacteria.

The physiological state of microbial communities was determined using the ratios of MUFA/SATFA and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy17:0+cy19:0/16:1 ω7c+18:1 ω7c) **(Frostegård and Bååth 1996)**.

Statistical analysis

Student's t-test was used to determine the significance of differences in the horizontal distribution of roots between the means of two independent samples. Biomass, root trait variables and PLFA proportions were analyzed with one- or two-way analyses of variance (ANOVA) according to each experiment. All treatments were regarded as fixed factors (species, root segregation, root exudates, concentration, interactions between species and root segregation, as well as interactions between species and concentration). Tukey's honest significant difference test was used for multiple comparisons when ANOVA terms were significant using SPSS 16.0 for Windows (SPSS Inc. Chicago, Illinois, USA). Principal component analysis (PCA) was applied separately to PLFA proportions to show relationships among soil samples in microbial compositions. Significant differences between treatments in ordination space were tested with a MANOVA on the principal component scores. PCA was performed with the STATISTICA software package, version 6.0 (Statsoft Inc., Tulsa, Oklahoma, USA).

Results

Effects of mixed-species planting on the growth of Chinese fir

Compared with the Chinese fir monoculture, the growth of Chinese fir was significantly enhanced by the presence of *E. decipiens* or *M. macclurei*, whereas the strongest inhibition occurred in the presence of *C. camphora*. Regardless of the broadleaf species, the effects of mixed planting were greater on the root growth than on the shoot growth of Chinese fir (Fig. 1). Furthermore, mixed planting with *M. macclurei* significantly increased the total root **occupation** area (Fig. 2A), total root length (Fig. 2B) and maximum root **amplitude** (Fig. 2C). When Chinese fir was planted with *E. decipiens*, the total root length increased (Fig. 2B). However, the total root length and maximum **root amplitude** were strongly inhibited when **Chinese** fir was planted with *C. camphora* (Fig. 2). In contrast, compared to the Chinese fir monoculture, mixed planting with *L. formosana* did not influence root growth parameters (Fig. 2). The maximum root depth did not differ significantly among treatments (Fig. 2D).

Root placement patterns of Chinese fir in the presence of broadleaf species

When Chinese fir and broadleaf trees grew together, Chinese fir adjusted the horizontal placement of its roots in response to its neighbors (Fig. S1). However, horizontal asymmetry in root length and root biomass showed different distributions in the presence of different broadleaf species. The roots of Chinese fir grew towards the neighboring *E. decipiens* or *M. macclurei*, but avoided growing towards *C. camphora* and were unaffected by neighboring Chinese fir or *L. formosana* (Fig. 3). Furthermore, Chinese fir root length and biomass increased near *E. decipiens* and *M. macclurei*, but were unchanged at the far end. In the presence of *C. camphora*, reductions were greater near *C. camphora* (Fig. 3).

Impact of root segregation on Chinese fir root growth

When broadleaf trees interacted with or without root segregation, the root growth of Chinese fir varied significantly depending on the broadleaf tree species. In all treatments, except for those completely separated with the plastic film, Chinese fir growth was enhanced by the presence of *E. decipiens* and *M. macclurei*, but reduced when grown with *C. camphora*. When considering interactions with or without root segregation in the Chinese fir monoculture and when mixed with *E. decipiens*, root segregation with nylon mesh led to an increase in Chinese fir root growth compared to

353 plants with root contact (Fig. 4). On the contrary, when grown mixed with *M.*
354 *macclurei* or *C. camphora*, the root biomass of Chinese fir significantly reduced by
355 segregation with nylon mesh (Fig. 4). A complete root segregation with plastic film
356 resulted in no variation in growth regardless of neighbor identities. The analysis of
357 variance revealed significant differences among root segregation patterns and tree
358 species.
359
360 *Effects of root exudates on Chinese fir growth*
361
362 Our results showed that the root exudates of *C. camphora* greatly inhibited the root
363 growth of Chinese fir even at low concentrations. Chinese fir root exudates also
364 inhibited the growth of its own seedlings when applied in a sufficient concentration
365 (Fig. 5). However, the inhibition disappeared when root exudates were diluted. On the
366 contrary, the growth of Chinese fir was stimulated by the root exudates of *E.*
367 *decipiens* and *M. macclurei* (Fig. 5). Their positive effects increased with elevating
368 concentrations. As for the effect of *L. formosana,* there was a slight increase in root
369 biomass. The analysis of variance revealed more significant differences among tree
370 species than among concentrations.
371
372 *Effects of broadleaf species on* ***the composition of main soil microbial groups*** *of*
373 *Chinese fir and their root exudate relations to soil microbiota*
374

375 Compared with the Chinese fir monoculture, mixed planting with *M. macclurei*
376 significantly increased the PLFAs of actinomycetes and saprophytic fungi in the
377 rhizosphere soil of Chinese fir under root segregation conditions (Table 1). Similarly,
378 *E. decipiens* induced a great increase in most PLFA parameters. When grown with *L.*
379 *formosana*, there were only small differences in most PLFA parameters. However,
380 when compared with mixed planting with *M. macclurei* and *E. decipiens*, *C.*
381 *camphora* resulted in significant reductions in SATFA, MUFA, PUFA, Gram (+),
382 Gram (-), non-specific bacteria, actinomycetes, total bacteria, saprophytic fungi and
383 total PLFAs in the Chinese fir rhizosphere (Table 1). PCA scores clearly distinguished
384 the PLFA composition of soil samples from the Chinese fir monoculture and mixed
385 planting with specific broadleaf species (MANOVA Wilks'λ, $P = 0.009$).
386
387 **The composition of main soil microbial groups** of Chinese fir was similar in mixed
388 planting with *M. macclurei* and *E. decipiens*, which, however, differed from that of
389 the Chinese fir monoculture and mixed planting with *L. formosana* and *C. camphora*.
390 Each group occupied a distinct ordination space. The first principal component (PC1
391 = 26.8%) and second principal component (PC2 = 15.8%) together accounted for
392 42.6% of the total variation (Fig. 6). To investigate further the relationships between
393 planting patterns and the **composition of main soil microbial groups** in different root
394 segregation conditions, root exudates were added into the soil to examine their impact
395 on the corresponding microbial community composition. Subsequently, signature lipid
396 biomarkers increased once root exudates of *E. decipiens* or *M. macclurei* were applied.

Specifically, root exudates of *M. macclurei* significantly increased soil bacteria, Gram (+), Gram (-), actinomycetes and total PLFAs. However, when compared with the control (distilled water), signature lipid biomarkers of total PLFAs, bacteria, Gram (+), Gram (-) and actinomycetes reduced when the root exudates of Chinese fir were applied. Similarly, the root exudates of *C. camphora* reduced these PLFA parameters, except for Gram (-). Furthermore, specific variation in soil microbiota driven by root exudates of different tree species occurred during early incubation periods (3 days), whereas no changes in soil PLFAs were observed after longer incubation periods (9 days), except for the root exudate application of Chinese fir (Fig 7).

Discussion

The present study provides evidence for mixed planting with broadleaf tree species affecting the growth of the conifer Chinese fir and interspecific interactions depending on the specific identity of neighbors. Furthermore, the effects of broadleaf species on Chinese fir are generated through belowground chemical interactions, where root exudates of the neighbors influence root placement patterns and alter **the composition of main soil microbial groups**. Many previous studies have shown that the neighbor identity influences plants' growth responses in a species-specific manner (Belter and Cahill 2015; Weidlich et al. 2018). Consistent with these studies, we found that *E. decipiens* and *M. macclurei* **promoted** the growth of Chinese fir, but *C. camphora* **inhibited** its growth. In addition, the impact on root growth was stronger than that on the aboveground parts. It has been shown previously as well that belowground plant interactions are stronger than aboveground interactions on the performance of coexisting plants (Wardle et al. 2004).

In the present study, we evaluated root interactions and root placement patterns

between broadleaf species and the conifer Chinese fir through window rhizobox and root segregation experiments. Here, the window- and segregation-based methods comprehensively demonstrated interactions between broadleaf species and Chinese fir at the root level. This study suggested that the distinct responses of Chinese fir roots **were** mediated through belowground interactions. Root growth was altered by segregation with nylon mesh but not when plastic film was used. The plastic film completely blocked belowground root and soil interactions between broadleaf trees and Chinese fir. In this case, the interactions **were** limited only to those that occur aboveground. Our results clearly showed that interaction effects between broadleaf trees and Chinese fir mainly occur belowground and not aboveground. Root segregation with nylon mesh was thought to ease the competition and, actually, Chinese fir growth was enhanced by the presence of *E. decipiens*. However, when Chinese fir was planted with *M. macclurei*, the positive interactions were reduced by nylon mesh segregation. On the other hand, root segregation led to a significantly increased inhibition by *C. camphora*. These contradictory results may be related to the biochemical plasticity of specific plants. Some plants can regulate their growth and the production of defence metabolites in response to neighboring plants and other environmental changes, resulting in morphological and chemical plasticity (Metlen et al. 2009; Kong et al. 2018). **Previously, Zhang et al. (2016b) have discovered that the root biomass of several weeds was reduced more even when the roots were segregated using 30 μm mesh that prevents physical contact but not potential chemical signals or microbial interactions with wheat.** The reason for this

463 phenomenon is that allelopathic wheat increases allelochemical secretion in response
464 to alterations in root-root interactions, leading to a significantly reduced weed
465 biomass.
466
467 In our study, root segregation altered the balance of root interactions between Chinese
468 fir and specific broadleaf species, such as *M. macclurei* or *C. camphora*. In this
469 scenario, the content and composition of bioactive molecules, produced and released
470 by corresponding broadleaf species, **may** vary depending on the root contact and lead
471 to a different performance in Chinese fir. Chemical interactions always occur between
472 plants growing together (Jose et al. 2006). Neighboring plants can exert chemical
473 effects, including allelopathy and allelobiosis, to influence plant survival and growth.
474 Allelopathy is generally considered to have a negative effect, while allelobiosis causes
475 a positive effect by donor plants through releasing **non-toxic signaling molecules** to
476 trigger stimulative responses in the recipient plants and to improve their fitness
477 (Glinwood et al. 2011; Kong et al. 2018). Each species may possess own biochemical
478 traits, which are under the genetic control of the host and exert distinct belowground
479 effects on neighboring plants (Tsunoda et al. 2017). In the present study, root exudates
480 from broadleaf trees mediated the root responses of Chinese fir in a species-specific
481 fashion. The impact of the root exudates of Chinese fir and *C. camphora* on Chinese
482 fir was negative, which indicated that allelochemicals present in root exudates inhibit
483 the growth of Chinese fir. However, it was surprising that the Chinese fir performance
484 was enhanced by a relatively low concentration of their own root exudates. Previous

485 studies have suggested that Chinese fir roots release cyclic dipeptides into soil to
486 hinder the natural regeneration and growth of Chinese fir, resulting in autotoxicity
487 (Kong et al. 2008; Chen et al. 2014). Interestingly, low concentrations of cyclic
488 dipeptides could result in improved survival and growth of Chinese fir (Xia et al.
489 2016).
490
491 Also, some **non-toxic signaling molecules** in root exudates can positively promote
492 the growth of Chinese fir. Its performance was enhanced by the root exudates from *M.*
493 *macclurei* and *E. decipiens*, and the facilitation effect was reduced when the
494 concentration of root exudates was lower. Several studies have reported the chemical
495 facilitation effects of root exudates on plant-plant interactions (Babikova et al. 2013;
496 Rasmann and Turlings 2016; Xia et al. 2016; Zhang et al. 2016a). Li et al. (2016)
497 showed that intercropped maize promotes faba bean growth, where the **non-toxic**
498 **signaling molecules** of maize root exudates enhance the flavonoid synthesis in faba
499 bean, stimulate nodulation, and increase nitrogen fixation. When investigating
500 mixed-species plantation forests, Yang et al. (2009) found that the growth of
501 Manchurian walnut (*Juglans mandshurica*) seedlings was inhibited by their own root
502 exudates but stimulated by larch (*Larix gmelini*) root exudates. The performance of
503 plants in mixed-species systems largely depends on belowground ecological
504 interactions (Jose et al. 2006; Lei et al. 2012). The effective chemicals in root
505 exudates can mediate these processes, such as preventing root growth, regulating root
506 placement patterns and shaping soil microbial communities; thus, subsequently,

affecting the characteristics of plants themselves and those of co-existing plants (Falik et al. 2005; Broeckling et al. 2008; Cesco et al. 2012; Xia et al. 2016).

In our study on root interactions between broadleaf species and Chinese fir, we found that Chinese fir **could** recognize the neighbor identity and alter root growth and placement patterns. Across all broadleaf trees tested, there was no consistent behavioural response to Chinese fir, resulting in three root placement patterns: intrusive, unresponsive and avoidance. The impact of root competition on root placement patterns in plant-plant interactions is well known (Bartelheimer et al. 2006; Novoplansky 2009; Cahill and McNickle 2011). Recently, much attention has focused on species-specific effects of allelochemicals and **non-toxic signaling molecules** on root behaviour (Semchenko et al. 2014; Asaduzzaman et al. 2016; Yang et al. 2018). In this study, we found that the application of *C. camphora* root exudates resulted in the root growth inhibition of Chinese fir, but root exudates from *M. macclurei* and *E. decipiens* stimulated Chinese fir growth. Furthermore, we suggest that allelochemicals from *C. camphora* are rhizosecreted and move into the bulk soil. To avoid such allelochemicals, Chinese fir roots tend to move towards locations not occupied by allelopathic *C. camphora* roots. By contrast, once *M. macclurei* or *E. decipiens* roots release compounds acting as **non-toxic signaling molecules** into the soil, the Chinese fir roots are inclined to increase growth towards releasing positions. Consequently, the clearly distinct allelochemicals and **non-toxic signaling molecules** may lead to different root placement patterns in Chinese fir.

It is noteworthy that a variety of mechanisms, including resource competition, frequency-dependent predation and different environmental factors, may affect plant-plant interactions (Chesson 2000; Matsushima et al. 2012). The performance of plants is ultimately the net result of positive and negative interactions among the involved species (Jose et al. 2006; Zhang et al. 2014). Although our results and hypotheses were not completely consistent, it is a fact that the role of chemical mechanisms, such as those mediated by root exudates, largely affect plant-plant interactions. However, our manipulative experiments took place in a greenhouse and the experimental period was relatively short. In nature, competition for resources potentially shapes plant traits and induces phytochemical variation (Metlen et al. 2009; Broz et al. 2010). Several studies have shown that the amount and composition of root exudates produced and released by plants are correlated with environmental factors (Kong et al. 2002; Watson and Carter 2008; Nakayama and Tateno 2018). As a result, further research is needed to clarify the relationships between environmental variation, chemical responses of plants and the ecological function of root exudates in the field. Plant-plant interactions shape soil microbial communities through nutrient competition and root exudate secretion (Broeckling et al. 2008; Guo et al. 2019).

In the current study, Chinese fir and broadleaf trees were segregated with 30 μm nylon mesh to prevent direct competition for nutrients. In this way, only chemical and

551 microbial interactions of the two plant species were allowed. **Any retention and**
552 **microbial degradation in soil should affect the concentration and final**
553 **destination of the moving bioactive molecules (Watson and Carter 2008; Xia et al.**
554 **2015). Li et al. (2013) found that there were negative relationships between the**
555 **mobility values of bioactive molecules and soil organic matter contents. In this**
556 **study, the soil we used has a low organic matter content and, thus, it may resist**
557 **soil adsorption to some extent, Adequate soil moisture would largely facilitate the**
558 **migration of bioactive molecules.**
559
560 **Ultimately,** we found a compositional shift in the soil microbial communities among
561 different species combinations. Furthermore, root exudates altered **the composition of**
562 **main soil microbial groups** in a species-specific manner. *M. macclurei* and *E.*
563 *decipiens* induced microbial shifts that were adequate for the growth of Chinese fir.
564 On the contrary, the application of root exudates from Chinese fir or *C. camphora*
565 **triggered the development of a soil microbial community that was unfavorable**
566 **for Chinese fir growth**. Each plant species is thought to select specific soil microbial
567 communities through **litter or** root exudates (Hartmann et al. 2009; **Ren et al. 2017;**
568 **Boyrahmadi and Raiesi 2018; Li et al. 2019**). Plants not only provide C for
569 microorganisms, but some plant species also contain unique allelochemicals or
570 **non-toxic signaling molecules** in their exudates (Rasmann and Turlings 2016).
571 **During the movement, soil microorganisms take advantage of bioactive**
572 **molecules as carbon substrates. In turn, bioactive molecules** regulate a plant's own

and other plants' soil microbial communities, resulting in a positive or negative soil feedback process (Li et al. 2014; Xia et al. 2016; Zhou et al. 2018).

Conclusions

Using a series of manipulative experiments, we found that the conifer Chinese fir shows different responses to the neighboring broadleaf trees. There was clear evidence for following mechanisms: (i) the influence is greater on the growth of Chinese fir roots than on the growth of shoots regardless of the broadleaf tree species; (ii) root placement patterns of Chinese fir vary in a species-specific manner; (iii) there are changes in the abundance of main soil microbial groups. Furthermore, root exudates may alter belowground ecological interactions. **This study did not clarify in detail, which compounds are responsible for the observed effects. Therefore, we are continuing our research to gain deeper insights into inter-specific interactions between Chinese fir and broadleaf tree species mediated by root exudates in mixed-species plantations. Such knowledge of interaction mechanisms would be helpful when planning reforestation programs to establish mixed-species plantation forests.**

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Figure legends

Figure 1 The performance of Chinese fir in the presence of broadleaf species. Bars with same letters are not significantly different from each other at $P < 0.05$, according to ANOVA, followed by Tukey HSD tests.

Figure 2 Effects of broadleaf species on root development of Chinese fir. Columns with the same letter are not significantly different at $P < 0.05$ according to ANOVA, followed by Tukey HSD tests.

Figure 3 Horizontal allocation of Chinese fir root length (A) and biomass (B) in response to broadleaf species. A left or right position from zero indicates that Chinese fir roots grow away or towards in relation to the roots of neighboring broadleaf trees. The asterisks indicate the significance of differences in Chinese fir root growth between two positions mentioned above based on one-sample t-tests, $*P < 0.05$, $**P < 0.01$. Bars with different letters denote significant differences in Chinese fir root

859 growth among different mixed-species treatments in each specific position at $P < 0.05$
860 according to ANOVA, followed by Tukey HSD tests.
861
862 **Figure 4** The root performance of Chinese fir grown with broadleaf species under
863 root contact or root segregation (nylon mesh or plastic film). Columns with the same
864 letter are not significantly different at $P < 0.05$ according to ANOVA, followed by
865 Tukey HSD tests
866
867 **Figure 5** Effects of the root exudates from Chinese fir or broadleaf species on the root
868 growth of Chinese fir seedlings. The root exudates are diluted 1/1 and 1/4 with
869 distilled water. Data in a column followed by the same letter are not significantly
870 different at P=0.05 according to ANOVA, followed by Tukey HSD tests.
871
872 **Figure 6** Principal component plot of **the main microbial groups** in the rhizosphere
873 of Chinese fir planted with specific broadleaf species or another Chinese fir. Data
874 used in the PCA plots are transformed using sample unit to represent the relative
875 abundance of each PLFA (nmole percentage of total PLFA).
876
877 **Figure 7** The total PLFAs, bacteria, fungi, Gram (+), Gram (-), and actinomycetes in
878 soil incubated with root exudates from different species with different incubation
879 times. The data are presented by mean ± sed. Data in a column followed by the same
880 letter are not significantly different at P=0.05, according to ANOVA, followed by

881 Tukey HSD tests.

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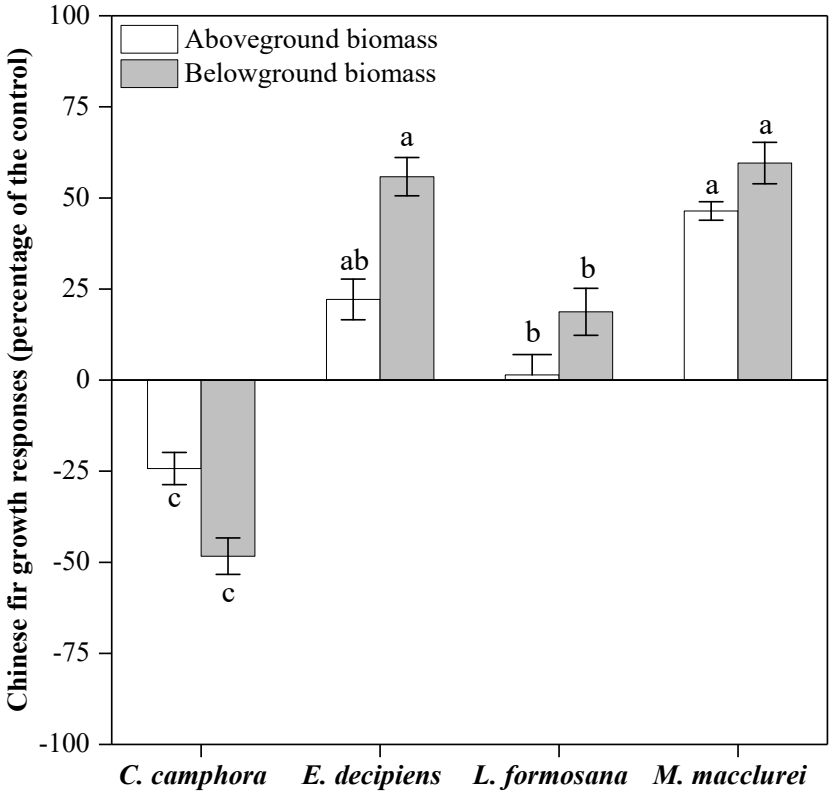
887 **Fig. 1**



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900 **Fig. 2**

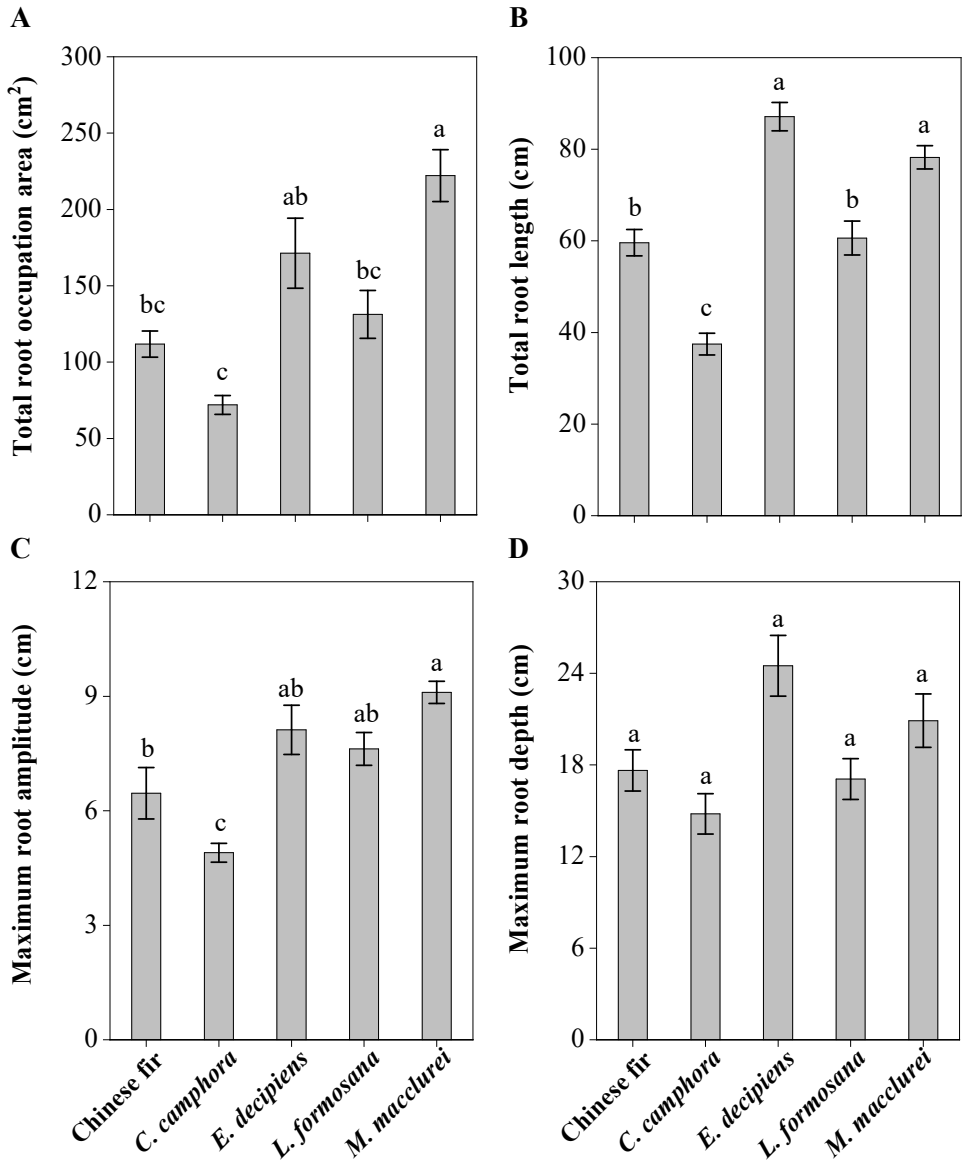

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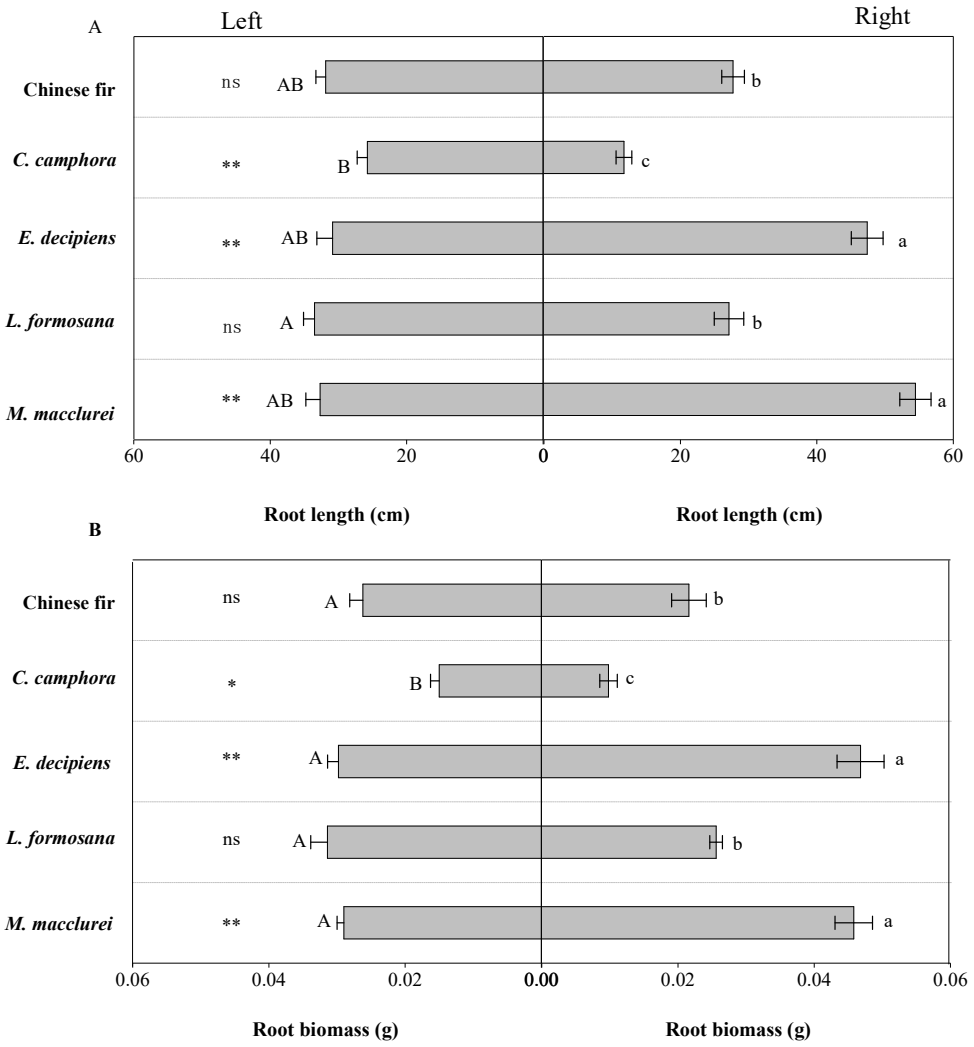
Fig. 3


A
Left
Right
Chinese fir
C. camphora
E. decipiens
L. formosana
M. macclurei
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Root length (cm)
Root length (cm)
B
Chinese fir
C. camphora
E. decipiens
L. formosana
M. macclurei
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Root biomass (g)
Root biomass (g)

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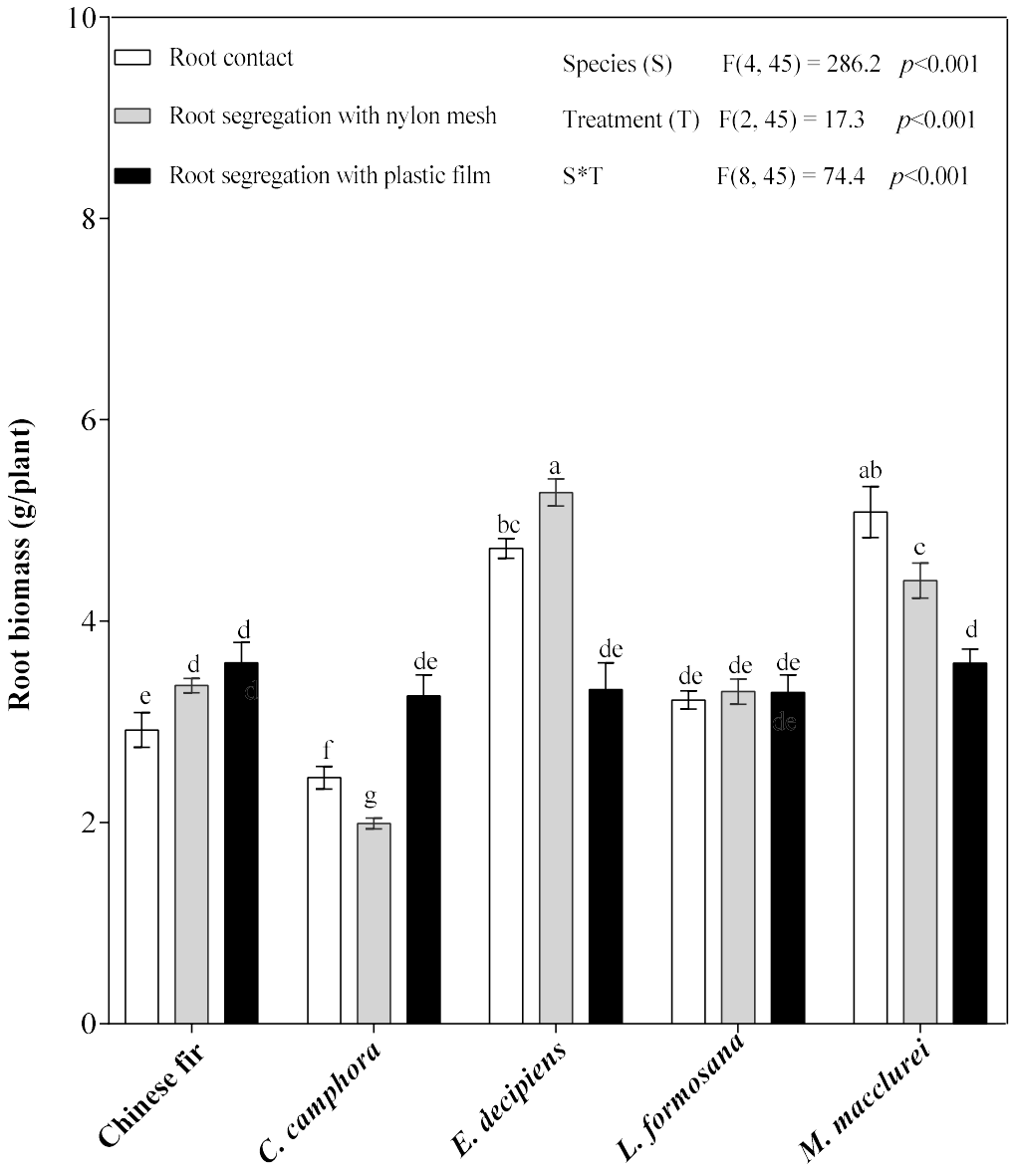
919 **Fig. 4**



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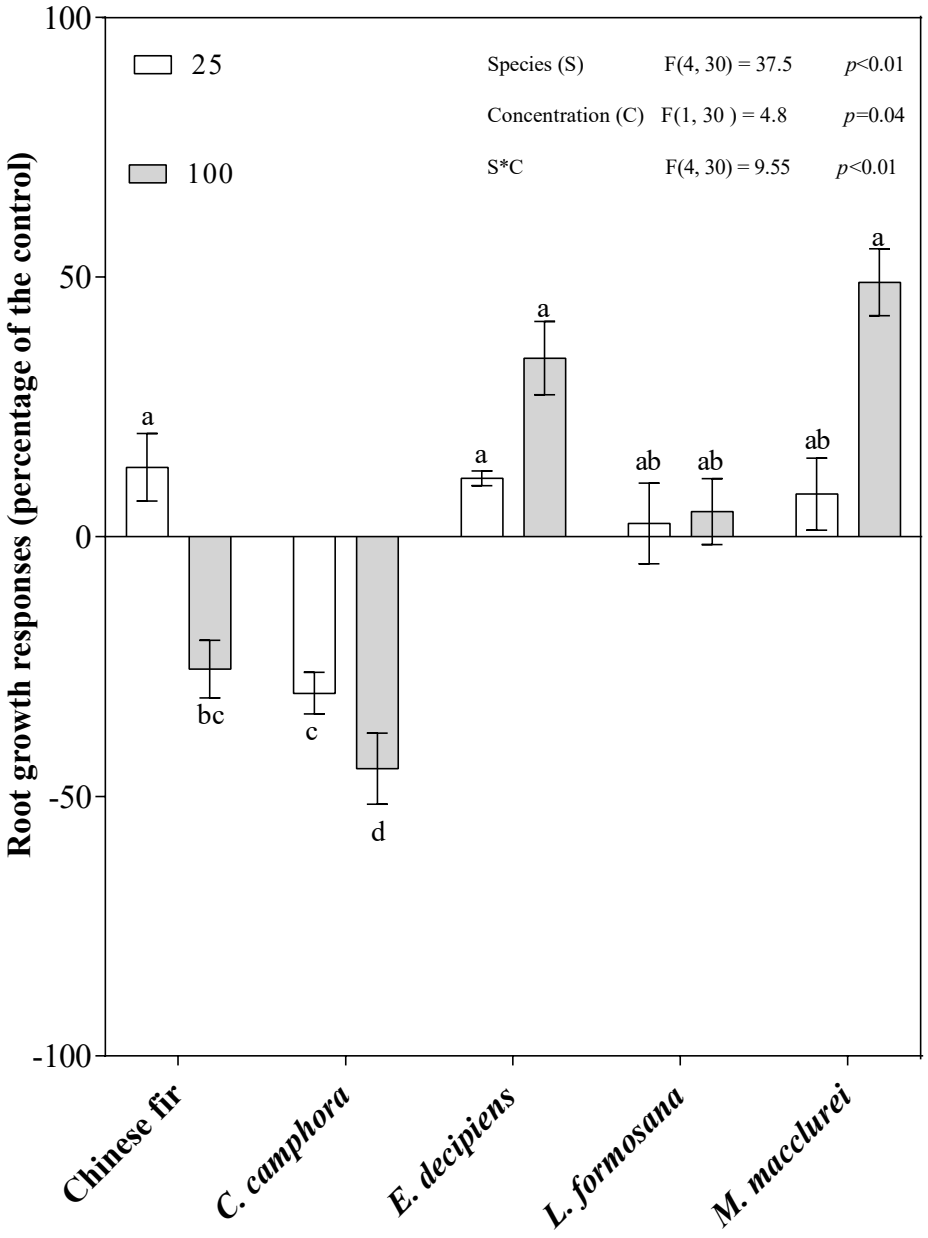
927 **Fig. 5**



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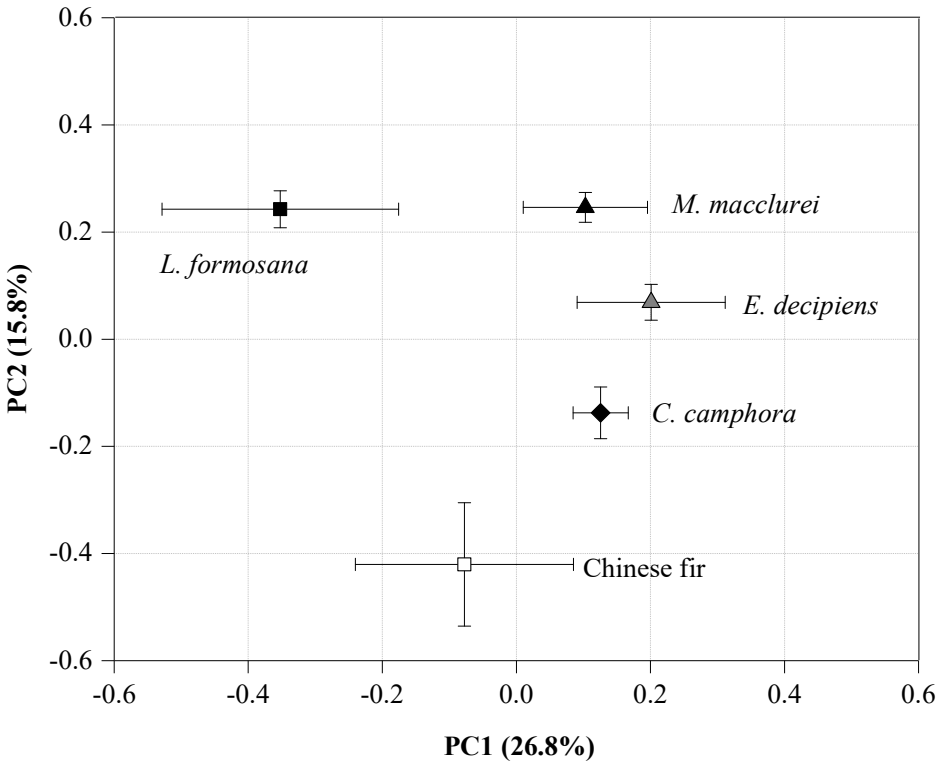

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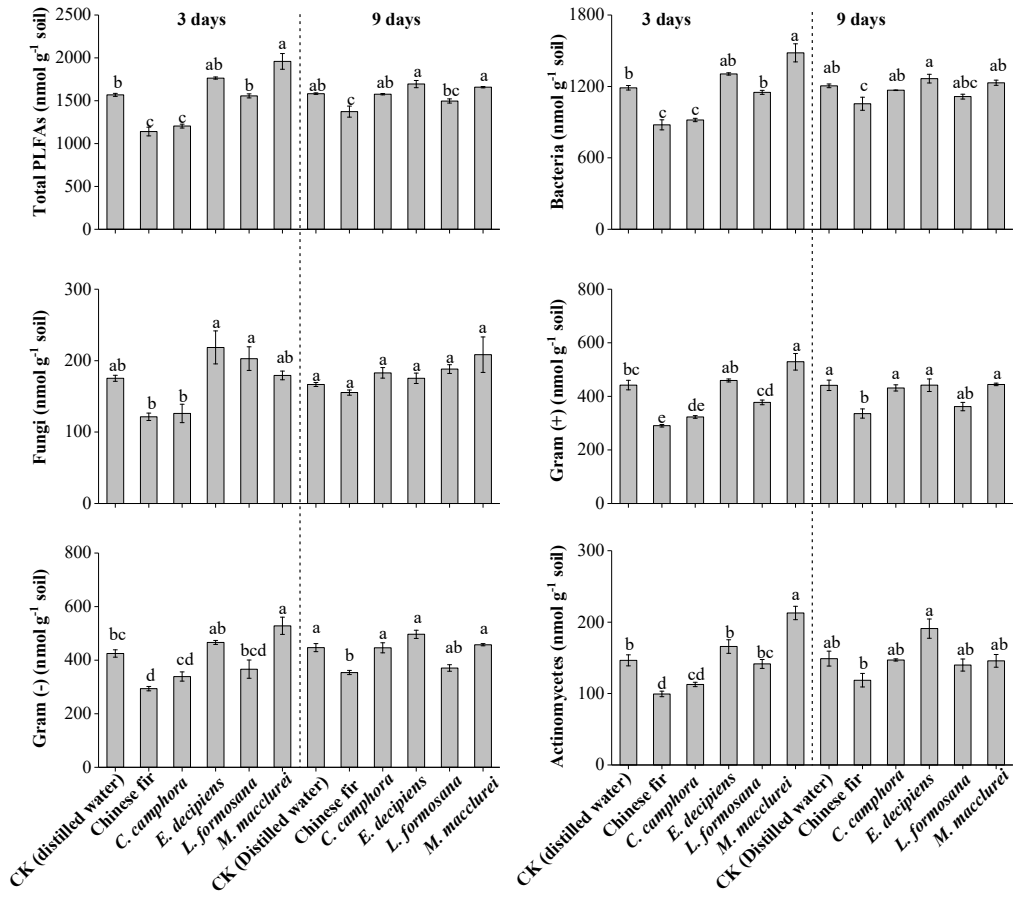
948 **Fig. 7**



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960 **Table 1** Soil PLFA concentrations (nmoles per gram dry soil) and selected microbial

961 community traits in the rhizosphere of Chinese fir planted with another Chinese fir or
962 specific broadleaf species.

	Chinese fir	*C. camphora*	*E. decipiens*	*L. formosana*	*M. macclurei*
SATFA	1015.61±33.39ab	766.56±51.87c	1224.28±58.71ab	988.33±12.39bc	1312.28±115.57a
MUFA	523.64±35.06ab	396±37.04b	620.02±20.56a	562.13±13.14ab	662.7±73.48a
PUFA	70.06±10.22ab	52.59±4.75b	100.92±3.57a	105.84±24.92a	110.4±9.45a
Gram (+)	410.81±9.74abc	315.48±23.45c	482.32±27.57ab	360.63±3.09bc	510.06±45.71a
Gram (-)	451.4±32.59ab	335.27±32.8b	538.36±25.37a	457.13±2.76ab	559.51±54.72a
non-specific bacteria	369.79±19.77ab	263.94±17.51b	435.69±14.59a	394.75±25.9a	482.82±46.28a
actinomycetes	144.97±6.17bc	117.9±8.61c	189.03±7.52ab	145.17±7.56bc	195.36±16.42a
total bacteria	1232.01±61.31ab	914.69±73.21b	1456.37±67.3a	1212.51±24.5ab	1552.39±145.5a
saprophytic fungi	163.1±9.8bc	132.66±11.94c	230.99±12.08ab	232.43±28.45ab	261.74±25.87a
AMF	69.24±4.67ab	49.9±1.7b	68.82±3.55ab	66.2±1.75ab	75.88±10.73a
total PLFAs	1609.31±57.15ab	1215.15±93.03b	1945.22±73.21a	1656.31±47.09ab	2085.37±195.52a
Gram (+): Gram (-)	0.92±0.05a	0.95±0.03a	0.9±0.01ab	0.79±0.01b	0.91±0.02ab
MUFA: SATFA	0.51±0.02a	0.52±0.02a	0.51±0.02a	0.57±0.01a	0.5±0.01a
cyc:prec	0.38±0.04a	0.4±0.02a	0.41±0.03a	0.34±0.03a	0.43±0.02a
fungi: bacteria	0.13±0.02a	0.14±0a	0.16±0.01a	0.19±0.02a	0.17±0.01a

963 For each parameter, values sharing the same letter are not significantly different
964 (P<0.05). SATFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA:
965 polyunsaturated fatty acids, cyc/prec: ratio of cyclopropyl PLFAs to their monoenoic
966 precursors.